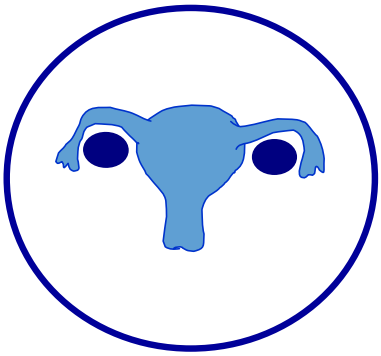
Ovarian carcinomas and borderline ovarian tumors

- molecular markers and operative treatment

Elin Ødegaard, MD



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PAPERS I-V

1. ACKNOWLEDGEMENTS

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Elin Ødegaard, January 2008

2. LIST OF PAPERS

Paper I:
Elin Ødegaard, Anne Cathrine Staff, Anton Langbrekke, Vibeke Engh, Mathias Onsrud.
Surgery of borderline tumors of the ovary: retrospective comparison of short-term outcome after laparoscopy or laparotomy.
Acta Obstetricia et Gynecologica 2007;86;620-626.

Paper II:
Elin Ødegaard, Anne Cathrine Staff, Janne Kærn, Vivi Ann Flørenes, Juri Kopolovic, Claes G. Tropé, Vera M. Abeler, Reuven Reich, Ben Davidson.
The AP-2γ transcription factor is upregulated in advanced-stage ovarian carcinoma.
Gynecologic Oncology 2006;100:462-468.

Paper III:
Elin Ødegaard, Anne Cathrine Staff, Vera M. Abeler, Juri Kopolovic, Mathias Onsrud, Philip Lazarovici, Ben Davidson.
The activated nerve growth factor receptor p-TrkA is selectively expressed in advanced-stage ovarian carcinoma.
Human Pathology 2007;38:140-146.

Paper IV:
Elin Ødegaard, Ben Davidson, Bente Vilming Elgaaen, Magne K. Fagerhol, Vibeke Engh, Mathias Onsrud, Anne Cathrine Staff.
Circulating calprotectin in ovarian carcinomas and borderline tumors of the ovary.
American Journal of Obstetrics and Gynecology, accepted for publication October 2007.

Paper V:
Elin Ødegaard, Ben Davidson, Vibeke Engh, Mathias Onsrud, Anne Cathrine Staff.
Assessment of endoglin and calprotectin as potential biomarkers in ovarian carcinoma and borderline tumors of the ovary.
American Journal of Obstetrics and Gynecology, accepted for publication December 2007.

3. ABBREVIATIONS

AC	adjuvant chemotherapy
AP-2 proteins	Activator protein-2, family of transcriptions factors
BOT	borderline ovarian tumor
BRAF	v-raf murine sarcoma viral oncogen homolog B1
BRCA	breast and ovarian cancer locus
CA 125	cancer antigen 125
CK (7 and 20)	cytokeratin 7 and 20
c-kit	one of the tyrosine kinase receptors
CRP	C-reactive protein
DAB	diaminobenzidine terahydrochloride
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Eng	endoglin (also named CD 105)
ERK	extra-cellular regulated kinase
FIGO	International Federation of Gynaecology and Obstetrics
GOG	Gynecologic Oncology Group
HNPCC	hereditary nonpolyposis colorectal cancer syndrome
HRAS	Harvay RAS
ICON	International Collaborative Ovarian Neoplasm
IHC	immunohistochemistry
JNK	c-jun amino-terminal kinase
kDa	kilo Dalton
KRAS	Kirsten RAS
MAPK	mitogen activated protein kinase
MMP	matrix metalloproteinases
MPSC	micropapillary serous carcinoma
MVD	microvessel density
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NRAS	neuroblastoma RAS
(PI3K)/AKT	the phosphoinositol-3-kinas/AKT pathway
P38	high-osmolarity glycerol response kinase
PMP	pseudomyxoma peritonei
PTEN	phosphatase and tensin homolog deleted from chromosome 10
RAS	family of cellular oncogenes
RMI	risk for malignancy index
RT-PCR	reverse transciptase polymerase chain reaction
SEng	soluble endoglin
SBT	serous borderline tumor
TGF	transforming growth factor
TMV	tissue microarray
TrkA	tropomyosin receptor kinase A
USO	unilateral salphingo-oophorectomy
WHO	World Health Organization
VEGF-A	vascular endothelial growth factor A
vWF	von Willebrand factor

4. INTRODUCTION

4.1 Cancer: uncontrolled cell growth

Cancer is a disease of uncontrolled cell growth. Cancer results from a series of acquired defects in the DNA that cause deregulation of the cell's growth processes. The damaged cell transforms from benign to malignant and becomes independent of normal regulatory signals. This transformed cell multiplies into a clone of malignant cells, eventually developing into a tumor (1). The malignant tumor infiltrates adjacent tissues and metastasizes. Cancer is a multi-step process, involving the progressive loss of control by the mutated cell with failure of DNA repair systems. Local invasion and distant metastasis are the main factors responsible for cancer-related morbidity and mortality. Both processes are functions of the complex interactions between tumor cells and the surrounding stroma, which are mediated by the production of a wide variety of molecules, among which matrix-degrading proteases, growth factors, adhesion molecules and angiogenic factors play a central role (2;3). Hanahan and Weinberg have summarized six essential acquired functional capabilities of cancer cells that drive the malignant growth, including self-sufficiency in growth signals, insensitiveness to anti-growth signals, capability to evade apoptosis (programmed cell death), limitless replication potential, capability to sustain angiogenesis, as well as capability to invade and metastasize (4). Metastatic disease is the cause of 90% of human cancer deaths (5).

4.2 Tumors of the ovary

4.2.1 Histological classification of epithelial tumors of the ovary

Neoplasms of surface epithelial origin account for about 65-70% of all primary ovarian tumors, but 90% of all malignant ovarian tumors (6). Epithelial tumors of the ovary are sub-classified according to morphology by WHO (World Health Organization) Classification of Tumors 2003 (7):

A. Serous tumors
Benign (cystadenoma, surface papilloma, adenofibroma)
Borderline tumor (papillary cystic tumor, surface papillary tumor, adenofibroma)
Malignant (adenocarcinoma, surface papillary adenocarcinoma, adenocarcinofibroma)

B. Mucinous tumors

Benign (cystadenoma, adenofibroma, cystadenofibroma)

Borderline tumor (intestinal type, endocervical like type)

Malignant (adenocarcinoma, surface papillary adenocarcinoma)

C. Endometrioid tumors including variants with squamous differentiation

Benign (cystadenoma, adenofibroma and cystadenofibroma)

Borderline tumor (cystic tumor, adenofibroma and cystadenofibroma)

Malignant (adenocarcinoma, adenocarcinofibroma, malignant mullerian mixed tumor, adenosarcoma, endometrioid stromal sarcoma, undifferentiated ovarian sarcoma)

D. Clear cell tumors

Benign (cystadenoma, adenofibroma)

Borderline tumor (cystic tumor, adenofibroma and cystadenofibroma)

Malignant (adenocarcinoma, adenocarcinofibroma)

E. Transitional cell tumors

Benign (Brenner tumor, metaplastic variant)

Borderline tumor (borderline Brenner tumor, proliferative variant)

Malignant (transitional cell carcinoma, malignant Brenner tumor)

F. Squamous cell tumors

Malignant (squamous cell carcinoma)

G. Mixed epithelial tumors

Benign

Borderline tumor

Malignant

H. Undifferentiated and unclassified tumors

Malignant (undifferentiated carcinomas, adenocarcinomas not otherwise specified)

4.2.2 Clinical staging, incidence and prognosis of ovarian cancer

Staging system of ovarian cancer according to FIGO (7)

Stage I: Tumor limited to the ovary

Stage IA: Tumor limited to one ovary, capsule intact, no tumor on the ovarian surface, no malignant cells in ascites or peritoneal washing

Stage 1B: Tumor limited to both ovaries, capsules intact, no tumor on the ovarian surface, no malignant cells in ascites or peritoneal washing

Stage IC: Tumor limited to one or both ovaries, with any of the following: capsule ruptured, tumor on the ovarian surface, malignant cells in ascites or peritoneal washings

Stage II: Tumor involves one or both ovaries with pelvic extension

Stage IIA: Extension and/or implants on uterus and/or tube(s), no malignant cells in ascites or peritoneal washings

Stage IIB: Extension to other pelvic tissues, no malignant cells in ascites or peritoneal washings

Stage IIC: Pelvic extension (IIA and IIB) with malignant cells in ascites or peritoneal washings

Stage III: Tumor involves one or both ovaries, with histologically confirmed peritoneal metastasis outside the pelvis and/or regional lymph node metastasis

Stage IIIA: Microscopic peritoneal metastasis beyond pelvis

Stage IIIB: Macroscopic peritoneal metastasis beyond the pelvis, 2 cm or less in greatest dimension

Stage IIIC: Peritoneal metastasis beyond the pelvis, more than 2 cm in greatest dimension and/or regional lymph node metastasis

Stage IV: Distant metastasis (excludes peritoneal metastasis). Pleural effusion must have positive cytology for stage IV

Note: Liver capsule metastasis is stage III, parenchymal liver metastasis equals stage IV

Clinical prognosis of ovarian cancer

Epithelial ovarian cancer is the leading cause of death from gynecological cancer in Western countries (8). Survival of ovarian cancer is closely related to the clinical stage at diagnosis (Figure 1). The incidence of ovarian cancer varies across nations, race and age. The highest incidence rates are reported from Northwestern Europe and North America, where the rates have remained almost constant over the last two to three decades (8). In Norway the incidence rate (average from 1996 to 2005) for ovarian cancer is 12.5 per 100.000 person-years (9). The incidence rate for borderline ovarian tumors (BOT) is 4.8 new cases per 100.000 (average 1995 to 2004) (10). Ovarian epithelial cancer has few early disease symptoms, most of the carcinomas (70%) are diagnosed when the disease is in advanced stage (8). The Cancer Registry of Norway has followed the survival rate for patients with borderline tumors only in recent years (Figure 2). Trimble et al. reported in a population-based study from USA (1988 to 1997), that 82% of the patients with BOT were in FIGO stage I, the 5 years relative survival was 99% (all stages) and that the 10-year relative survival was 95% (all stages). A study by Bjørge et al. (11) from Norway (1970 to 1993) found the age-adjusted 5-year survival rate for BOT (all stages) to be 93%. Seidman and Kurman summarized 97 reports, including a total of 4.129 women with serous BOT tumors, and showed a disease-specific survival rate of 99.5% (mean follow-up of 7 years) for stage I disease and 95.3% for stage III disease (12). Women with BOT are approximately 10 to 15 years younger than women with invasive epithelial ovarian cancer (13).

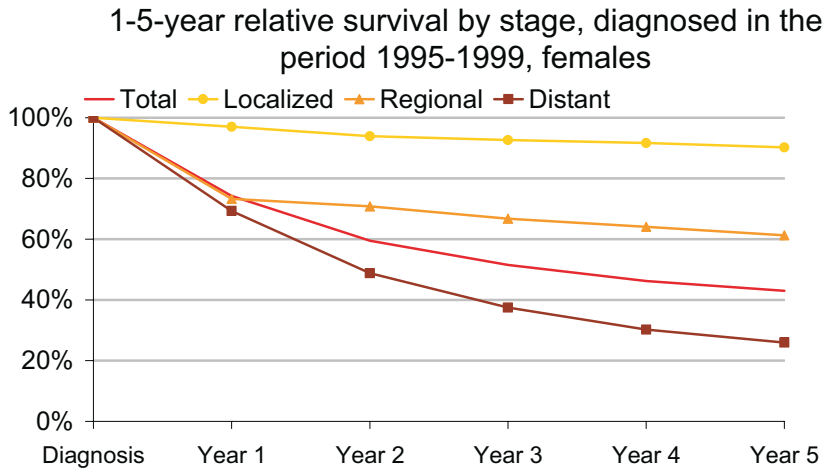

Figure 1: 1-5 years survival by stage, for women in Norway with invasive epithelial ovarian cancer diagnosed from 1995 to 1999 (Data from The Cancer Registry of Norway (10)). Red line shows the total survival, all stages.

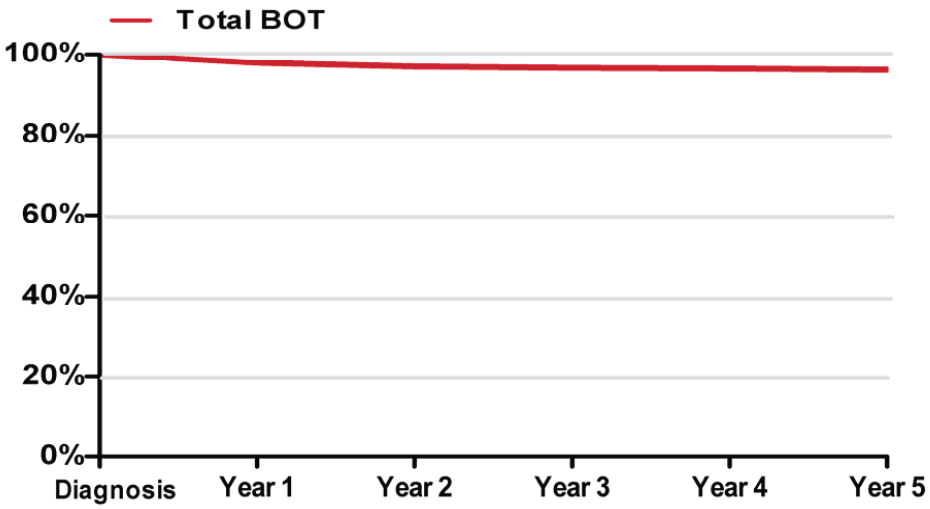

Figure 2: 1-5 year survival rate by stage, for women in Norway with BOT diagnosed from 1996 to 2000 (Figure by Elin Ødegaard, based on data from The Cancer Registry of Norway). BOT is associated with much better prognosis compared to invasive ovarian cancer. Red line shows the total survival, all stages.

4.2.3 Borderline ovarian tumors (BOT) versus invasive carcinoma

Borderline ovarian tumors (BOT) versus invasive carcinoma of the ovary borderline ovarian tumors, also named tumors of low malignant potential, are distinct from invasive ovarian malignancy. BOTs have a much better prognosis (Figure 1 and 2). Histologically, BOTs are characterized by epithelial hyperplasia and nuclear atypia, as well as by variable and usually minimal mitotic activity, and no destructive stromal invasion (7;14). As described in 4.2.1, BOTs can be divided histologically as serous (50%), mucinous (46%), or mixed, endometrioid, clear cell or Brenner tumors (the last 4 groups comprising 4% of BOTs) (15).

Serous borderline

There is a very high incidence of bilaterality in serous BOT (26-66%) (16).

Microinvasion

Although evidence of stromal invasion negate a diagnosis of borderline malignancy there have, nevertheless, been several reports of "microinvasion" (defined as one or more foci, and none exceeding more than $10mm^2$, (7)) of the stroma of both serous and mucinous tumors which otherwise showed the typical features of neoplasm of borderline malignancy (16). Such neoplasms follow the same clinical course and have the same prognosis as borderline ovarian tumors without microinvasion (16). Conceptually, neoplasms with microinvasion are not of borderline malignancy but clinically they are not adenocarcinomas (16), and when the foci of microinvasion is less than $10mm^2$, they are classified as BOT (7).

Micropapillary pattern

Serous borderline tumors may have two distinct different histological patterns in the same neoplasm. Tumors with micropapillary growth pattern (defined as foci <5mm, (17)) present more frequently as bilateral tumors and are more often associated with peritoneal implants than typical serous BOT (12;18-21). The presence of a significant micropapillary component in a BOT therefore needs to be specified in the pathology report (22). Some authors have proposed that this pattern of growth should be designated "micropapillary serous carcinoma" (MPSC) (17), because of its frequent association with aggressive behavior, but others do not share this view and prefer the designation serous borderline tumor (SBT) with micropapillary pattern (19-21). Micropapillary tumors behave aggressively if they are associated with invasive implants (19).

Invasive/ noninvasive implants

There has been, over the years, much argument as to whether the extraovarian lesions represent true metastases or whether they are independent of the ovarian neoplasm and develop in situ within the secondary Mullerian system, i.e. the submesothelial connective tissue (16). Morice et al. evaluated 80 patients with advanced serous BOT and concluded that the only prognostic factor for evolution to invasive disease was the type of peritoneal implants; 31% of patients with invasive implants developed invasive disease in a 5-year period, as compared to only 2% of patients with non-invasive implants (23). Non-invasive implants have almost no negative influence on the 10-year survival. The invasive implants are associated with a poor prognosis: more than 50% are associated with recurrence, and the 10-year survival rate is only about 35%. Invasive implants are usually localized in the omentum, and the tumor DNA ploidy is often aneuploid (7).

Mucinous borderline tumors

Two quite different types of mucinous BOT exist; the mucinous epithelium can be of gastrointestinal or of endocervical type (85% vs 15% of mucinous BOTs) (14). Pseudomyxoma peritonei (PMP) is a clinical term used to describe the finding of abundant gelatinous or mucoid material surrounded by fibrous tissue in the abdominal cavity (14). In patients with a mucinous BOT and PMP, the appendix is also involved in 60% of the cases (24;25). More than 80% of invasive mucinous carcinomas have components of mucinous borderline tumors and mucinous cystadenoma or both, suggesting a progression from benign to malignant neoplasia (14), a hypothesis supported by recent molecular studies (26-28). The majority of investigators believe that mucinous ovarian tumor(s) in patients with PMP are metastases from intestinal tumors where the primary tumor is most often located in the appendix (7). Primary ovarian mucinous tumors most frequently co-express cytokeratin 7 (CK7) and cytokeratin 20 (CK20), whereas metastatic tumors of gastrointestinal origin are predominantly or exclusively CK20 positive.

Somatic genetics in borderline ovarian tumors versus invasive carcinomas

The pattern of genetic alterations described in serous BOTs differs from that of invasive ovarian carcinomas. P53 mutations are most often absent in typical SBT (serous borderline tumors) and micropapillary SBT, but are present in up to 88% of invasive ovarian serous carcinoma (7;29;30). Recent studies strongly suggest that the sequence of malignant transformation from benign and borderline mucinous tumors to carcinoma represents

transitional stages of mucinous carcinogenesis. There are 3 types of RAS oncogenes (K,N and H), and mucinous BOTs have a higher frequency of KRAS mutation than that of mucinous cystadenomas, but a lower rate than that of mucinous carcinomas (7;26-28;31).

In general, the pathogenesis of ovarian carcinomas is unknown and there is a lack of a tumor progression model. Shih and Kurman have described a tumor progression model based on clinicopathological and molecular studies (32;33). They divide epithelial ovarian tumors into broad categories, type I and type II tumors, corresponding to two main pathways of tumorigenesis. Type I tumors are composed of mucinous carcinomas, endometrioid carcinomas, clear cell carcinomas, malignant Brenner carcinomas and low-grade serous carcinomas. Type I tumors are associated with low cellular proliferation and molecular changes such as mutation in BRAF and KRAS oncogenes for serous carcinoma, KRAS mutations for mucinous tumors, β-catenin and PTEN mutation for endometrioid tumors. In Type II tumors there are often TP53 mutations and TP53 amplification, overexpression of the HER2/neu gene and chromosomal instability. Shih and Kurman argue that type I carcinomas arise stepwise from BOTs. Type II tumors include high-grade serous carcinomas, malignant mixed mesodermal tumors (carcinosarcomas), and undifferentiated carcinomas. Shih and Kurman propose that type II carcinomas have no precursor lesion and develop de novo from surface epithelium of the ovary (32;33).

Hereditary ovarian cancer

Two genes have been identified that are associated with hereditary breast/ovarian cancer syndrome, BRCA1 and BRCA2 (breast and ovarian cancer locus 1 and 2). Mutation in these genes predisposes women mainly to breast cancer, but also to ovarian cancer. For female carriers of BRCA1 or BRCA2 mutations, the risk of developing ovarian cancer (by 70 years of age) is between 15% and 66% (34;35). The risk for BRCA2 mutation carriers is on average less than for BRCA1 (36) mutation carriers. For women with hereditary nonpolyposis colorectal cancer syndrome (HNPCC), the cumulative lifetime risk for ovarian cancer may be as high as 10% (37;38).

4.2.4 Clinical diagnosis of ovarian cancer

Gynecological examination, ultrasound and CT and /or MR together with serum CA 125 concentration are used today in diagnosing ovarian cancer. Screening for ovarian cancer in

the general population is not currently recommended (39). The sensitivity of CA 125 in early-stage ovarian cancer is low, and CA 125 also lacks high enough specificity to be recommended alone in a screening program of the general population. Many benign condition are associated with elevated CA 125 serum concentrations (40;41). Several studies aiming at identifying new biomarkers are ongoing, and in 2012 the results of two large screening trials will be available (42).

4.2.5 Treatment of ovarian cancer

Fertility-sparing surgery

In selected patients with child-bearing wish, who have stage IA and grade 1 or grade 2 ovarian carcinomas, unilateral salpingo-oophorectomy (USO) with inspection of the remaining ovary and proper staging is an option, with low risk of recurrence (43). In a recent meta-analysis of 282 patients treated conservatively for invasive ovarian carcinomas, 113 became pregnant, and 87 had term deliveries. Thirty-three patients (12%) developed ovarian carcinoma recurrence, and there were 16 disease-related deaths (4%) (44). For women with BOT, a meta-analysis with 1483 patients found 196 pregnancies and 111 relapses after USO (7.5%) (44). One patient who had disease-related death was not staged correctly (FIGO stage unknown) (45).

Optimal surgical staging for borderline ovarian tumors

Surgical removal of BOT is the cornerstone in the treatment (13). BOT affects younger women than invasive carcinomas, and preserving fertility is frequently an issue. Conservative surgery is defined as surgical preservation of uterus and at least a part of one ovary to preserve fertility. Radical surgery includes total abdominal hysterectomy and bilateral salphingo-oophorectomy (15). Proper staging for BOTs, both conservative and radical approach, includes exploration of the entire abdominal cavity, cytology from ascites or peritoneal washing, omentectomy, multiple peritoneal biopsies and resection of possible implants and appendectomy (only when there is a mucinous BOT) (15). Women with aneuploid BOT have increased risk of dying of the disease (46) and should be radically operated (47). Lymphadenectomy is not recommended in women with BOTs (15;47), as the recurrence rate and survival rate for patients are similar with and without this procedure (12;48).

Primary surgical staging for ovarian carcinomas

Surgery is the initial treatment of ovarian cancer, where removing as much as possible tumor volume is essential for survival (49). Surgery is also important for a correct histological diagnosis and for a correct surgical FIGO staging (50). FIGO staging includes cytology from ascites or peritoneal washing, omentectomy, complete removal of the tumor, lymph node sampling, hysterectomy and bilateral salphingo-oophorectomy (the latter two in patients not interested in or suitable for fertility-sparing surgery). Bilateral para-aortic and pelvic lymph node sampling is important in early-stage ovarian cancer. Among women with a clinically early-stage (FIGO stage I) ovarian cancer, 5-25% have lymph node metastasis (51-54). The incidence of positive para-aortic nodes in stage I ovarian carcinomas was reported to be 18% in a study by Chen and Lee (51).

Primary cytoreduction of advanced-stage ovarian carcinomas

Cytoreductive surgery for ovarian cancer treatment was first introduced in the 1930s by Meigs (55). In 1975, Griffiths published a landmark paper describing the impact of residual disease on survival (56). A meta-analysis on the effect of maximal cytoreductive surgery, removing as much tumor volume as possible, showed a statistically significant positive correlation between percent maximal cytoreduction and log median survival time (49). A consensus statement on management of ovarian cancer concluded in 2004 that maximal surgical effort at cytoreduction with the goal of no residual disease was the aim and when cytoreductive surgery is not possible initially, it should be considered in patients who do not have progressive disease after 3-5 cycles of chemotherapy (50).

Chemotherapy

The current standard treatment for advanced-stage ovarian carcinomas is a combination of paclitaxel and carboplatin given adjuvant to surgery (57-59). Adjuvant chemotherapy (AC) for early-stage ovarian cancer is controversial (60). Patients who are staged incompletely at primary operation should be completely surgically staged (including lymph node sampling) before a final decision can be made regarding the need for AC in this group. Clinical FIGO stage and sub-stage, DNA ploidy and preoperative CA 125 should also be taken into consideration when making this decision (60).

Targeted therapy in ovarian cancer

Targeted therapy has been shown to prolong patient life in some carcinomas, most convincingly in breast cancer. Breast cancer patients whose tumors overexpress HER2/neu are now treated with a monoclonal antibody (trastuzumab) targeting this receptor (61). In ovarian cancer, targeted therapy is currently evaluated through angiogenesis inhibition. Bevacizumab is a monoclonal antibody blocking the VEGF-A receptor, evaluated in breast cancer therapy. Initial therapy of metastatic breast cancer with paclitaxel plus bevacizumab prolongs progression-free survival, but not overall survival, as compared with paclitaxel alone (62). Today, there are two ongoing phase III trials with targeted therapy for ovarian cancer initiated by the Gynecologic Oncology Group (GOG 218) and International Collaborative Ovarian Neoplasm (ICON 7) (63). Both trials include standard carboplatin and paclitaxel treatment, together with bevacizumab, as front line therapy in ovarian cancer, in patients with newly diagnosed disease (64). GOG 218 is a three-arm, placebo-controlled trial, for patients with stage III-IV disease. ICON7 include all patients with ovarian cancer (also early-stage, high-risk) and is a two-arm trial without placebo, with 6 cycles of bevacizumab versus extended 12 cycles, the end point being progression free survival (63).

4.3 Biomarkers of ovarian tumors

A biomarker is defined as a measure indicator of a specific biological state, particularly one relevant to the risk of the presence or the stage of a disease (65). Biomarkers can be used clinically to screen for, diagnose or monitor the activity of diseases and as a guide for molecular targeted therapy or in assessment of therapeutic response (66).

The ovarian cancer biomarker CA 125 was first identified in 1981 (67), and is a large membrane-bound glycoprotein whose biological function remains unknown (68). CA 125 is the epithelial ovarian cancer biomarker of practical clinical use today, used to monitor response to chemotherapy, to detect disease recurrence and to distinguish malignant from benign pelvic masses (69). Jacobs et al. developed a risk of malignancy index incorporating CA 125, ultrasound and menopausal status that will estimate the probability of malignant potential for pelvic mass with a sensitivity of 85% and a specificity of 97% (70). Unfortunately, CA 125 is frequently elevated in noncancerous conditions, such as pregnancy, endometriosis, uterine fibroids, liver diseases and benign ovarian cysts, and in other malignancy, such as colon, uterine, fallopian, gastric and pancreatic cancers (71). CA

125 has low sensitivity for early-stage ovarian carcinomas (41), and CA 125 is not recommended as a screening marker in women who are not at high risk or do have specific symptoms of the disease (72).

Early-stage cancer lesions are small in tissue volume, thus limiting the biomarker production rate into a level well below the threshold of detection in circulation by most diagnostic platforms today. Low biomarker concentrations, especially for early-stage, reduce test sensitivity (71). Epithelial ovarian cancer is a very heterogeneous group of tumors as shown earlier in the introduction, with different genetic alterations and potential growth pathways.

Biomarkers can be measured in the circulation as well as at tissue level. Molecular markers can be detected on chromosomal level such as mutation (TP53), deletion (TP53 and TP16) or amplification (AKT2 and HER2/NEU)) of tumor suppressor genes or oncogenes. There are several classes of molecules involved in growth stimulatory pathways, including growth factors, transcription factors, and cell cycle proteins. These biomarkers can be measured on gene level (e.g. by microarray or fluorescent in situ hybridization), on mRNA level (e.g. by in situ hybridization, northern blot or RT-PCR) or on protein level (e.g. by immunohistochemistry or proteomics). There have been numerous studies of the prognostic impact of molecular markers for ovarian cancer. However, these studies have not yet resulted in the implementation of new molecular markers for diagnosis and follow-up.

4.4 Biomarkers explored in this thesis

4.4.1 AP-2γ and c-kit

The control of the gene expression is complex. Gene expression with transcription of the gene into mRNA is initiated by binding of transcription factors to the promoter. Several transcription factors are tissue specific. Activator protein-2 (AP-2) transcription factors are involved in the regulation of cell proliferation, differentiation, apoptosis and carcinogenesis (73-75). AP-2 transcription factors represent a family of five closely related proteins, termed α-ε, which are encoded by distinct genes (74). AP-2α, AP-2β and AP-2γ are the most well known members. These transcription factors are involved in cancer proliferation by directly activating the promoter of several central growth- and differentiation-related genes such as

$p21^{WAF1}$ (76), ER-α (77) and HER2/neu (73). In studies of melanoma cell lines, loss of AP-2α expression has been associated with several invasion- and metastasis-promoting events such as overexpression of the cell adhesion molecule MCAM/MUC18 and protease-activated receptor (PAR)-1, as well as downregulation of the tyrosine kinase receptor c-kit (78-80). An earlier study showed c-kit to be infrequently expressed in ovarian carcinomas (81).

The exact role of AP-2 transcription factors in human cancer development is not entirely established. However, there is growing evidence that the net effect of whether the cell proliferates, undergoes apoptosis or differentiates may partly be dependent on the balance between different AP-2 proteins (82;83). The role of AP-2γ may differ from that suggested for AP-2α (83). Different tumors have different expression of AP-2α and AP-2γ. AP-2γ also plays an essential role in early embryogenesis. Transgenic embryos lacking AP-2γ die soon after implantation (84;85). In breast cancer, the chromosomal locus of AP-2γ gene is often amplified, and this gene amplification is associated with poor prognosis (86). In HER2/neu overexpressing transgenic mice, AP-2γ expression is initially delayed, but when tumor growth is accelerated, AP-2γ expression increases (82). The only published study of AP-2 expression in ovarian carcinoma, to the best of our knowledge, analyzed AP-2α. In this study, nuclear AP-2α expression and absence of cytoplasmatic staining predicted worse prognosis (87).

4.4.2 Nerve growth factor receptors and intracellular signaling

Neurotrophins are a family of growth factors, in which nerve growth factor (NGF) is the prototype molecule. Their biological functions are mediated through binding to two classes of cell surface receptors, to the high-affinity tyrosine kinase receptor tropomyosin receptor kinase A (TrkA) and the low affinity neurotrophin receptor p75 a member of the tumor necrosis family (88). As for many other growth factors, dysregulation of neurotrophin signal transduction is found in a number of tumors where they can contribute to malignant transformation (89). TrkA was originally discovered as a colon carcinoma oncogene (90). Rearrangement or mutation of the TrkA gene, resulting in constitutive activation of the receptor, has been observed in colon carcinomas, thyroid papillary carcinomas, as well as in acute myeloid leukemia (91). Overexpression of TrkA in different tumors, including

prostate, pancreas, thyroid, lung, breast and ovarian carcinoma, and in malignant melanoma, is associated with poor prognosis (92-98).

The evidence regarding the biological role of p75 in human cancer is more limited. Frequent expression was seen in some neural and soft tissue tumors, while most carcinomas were negative (99). Furthermore, p75 expression has been found to be reduced in carcinomas compared to non-neoplastic tissue (100), although the latter finding was not confirmed in an additional study (101).

NGF ligand binding to TrkA receptors activates TrkA autophosphorylation at several sites (102;103). Recent studies of TrkA signaling in different cancers suggest that TrkA engagements leads to the activation of a variety of intracellular pathways, such as the mitogen-activated protein kinase (MAPK) pathway and the phosphoinositol-3-kinas (PI3K)/AKT pathway (103-105).The mitogen-activated protein kinase (MAPK) intracellular signaling is a four-kinase component cassette, in which each kinase activates the following kinase substrate through a complex network, enabling the cell to maintain diversity and specificity while responding to various extracellular stimuli (106). The final level consists of extra-cellular regulated kinase (ERK), c-jun amino-terminal kinase (JNK), and the high-osmolarity glycerol response kinase (p38). JNK and p38 are known to be activated by stress-related stimuli, and are thought to largely mediate apoptotic signals. ERK members are largely activated by growth factor signals, as those mediated by receptor tyrosine kinase, the net result being growth, differentiation, and proliferation (107). Givant-Horwitz et al. reported three activated forms of MAPK to be predictors of improved clinical outcome in effusions from serous ovarian carcinoma patients (106). In primary ovarian carcinomas, p-ERK is more frequently expressed in low-grade compared with high-grade tumors and correlates with better survival (108).

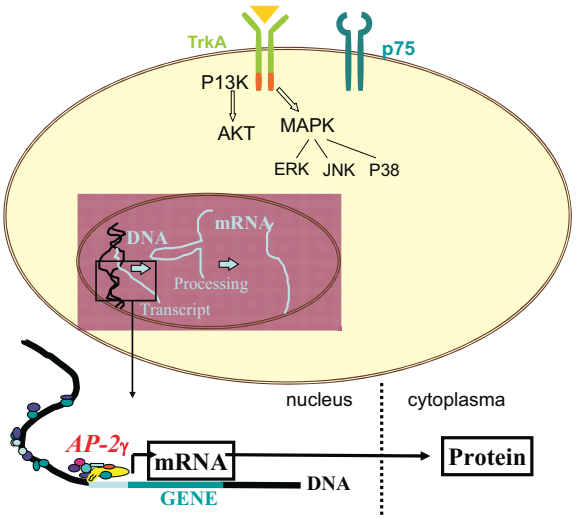

Figure 3: Factors explored in the thesis by immunohistochemistry.

Growth factors bind to the two cell surface membrane receptors explored in this thesis, TrkA or p75. TrkA receptors carry thyrosine kinase domains on their cytoplasmatic side. Growth factor binding to the receptor leads to phosphorylation of parts of intracellular pathways, such as MAPK and/or p13/AKT pathways (103-105). Activation of MAPK intracellular pathways, mediates cell proliferation, apoptosis and differentiation (107).
Ap-2γ transcription factor is involved in tumor proliferation by directly activating the promoter of several central proliferation related genes (73;76;77).

4.4.3 Calprotectin

Already in 1863, Rudolf Virchow noted the presence of leukocytes in neoplastic tissues and made a connection between inflammation and cancer (109). Since then, many experimental studies and clinical observations have linked cancer and inflammation (reviewed in (109;110)). The calprotectin protein is an inflammation marker, and has been associated with some forms of cancer.

Human leukocyte protein L1, later named calprotectin, was first described in 1980 by Fagerhol et al. (111). Calprotectin is a protein mainly found in granulocytes and monocytes,

it is a major calcium- and zinc-binding protein. Calprotectin is released by activated neutrophils (111;112). This 36.5 kD heterodimer protein molecule contains two 13.3 kD heavy (L1 heavy or MRP14) and one 8.2 kD light (L1 light or MRP 8) polypeptide chains (113). Each chain can bind two calcium ions. Other names for calprotectin include MRP8/MRP14 (114;115), 27E10 antigen (115), S100A8/S100A9 (116;117) complex and calgranulin (118;119).

Calprotectin plays a role in various physiological functions such as inflammatory processes, inhibition of cell proliferation, and in the neutrophil defense against bacterial infections (120). Apoptosis (programmed cell death) is linked with many pathological conditions such as inflammation and tumor growth. Calprotectin has cytokine like effects (121;122) including a proinflammatory cytokine function (117), as well as a chemotactic factor activity (123).

Matrix metalloproteinases (MMPs), family of zinc-dependent proteases, are able to break down extracellular matrix (ECM). MMPs are important in many normal biological processes, including embryonic development, angiogenesis, and wound healing, as well as being important in pathological processes such as inflammation and cancer (124). The metalloproteinases are totally dependent on zinc for their enzymatic activities (124) and Isaksen and Fagerhol demonstrated that calprotectin inhibits MMPs by competing with these enzymes for zinc (125). Among the MMPs that can be inhibited is collagenase B (MMP-9), known to be important during angiogenesis and invasive tumor growth.

Calprotectin is a member of the S100 protein family. The S100 genes are located in a gene cluster on chromosome 1q21 (126;127). This region has been identified as a target region for transcriptional activation in common human epithelial malignancies (128;129). S1008A and S1009A genes are overexpressed in gastric cancer, as demonstrated by RT -PCR on frozen tissue (130). Immunohistochemical investigations have shown that S100A9 protein is overexpressed in hepatocellular carcinomas, pulmonary adenocarcinomas, and invasive ductal carcinomas of the breast (131-133). In these tumors, elevated expression of S100A9 correlated with poor differentiation. Calprotectin has been shown to be elevated in the circulation (serum) and in fecal specimens from patient with colorectal carcinomas (134), and in serum as well as in the primary tumor in prostate cancer (116).

To our knowledge, the only published study of calprotectin expression in ovarian cancer was performed using gel electrophoresis. In this study, Ott et al. demonstrated elevated calprotectin concentration in ovarian cystic fluid as well as serum in women with ovarian cancer (n=11) compared to benign ovarian cysts (119).

The mechanisms behind the elevated plasma calprotectin levels and the exact roles of calprotectin in cancer are at present unclear.

4.4.4 Endoglin

Endoglin (Eng), also named CD105, is a cell-surface co-receptor for TGF (transforming growth factor)-β1 and -3, and is highly expressed on endothelial cells. The TGF-β superfamily consists of growth factors that govern a wide range of cellular functions, such as cell growth, differentiation, and migration (135). The TGF-β actions are mediated through intracellular signaling mediators, known as Smads (135), which alter transcriptional responses (136). Endoglin modulates the actions of for TGF-β family of growth factors, without interfering with ligand binding (137), and has been described as proliferation-associated antigen of leukemia and endothelial cells (138). TGF-β inhibits endothelial cell proliferation, migration and formation of microvessels. Cell-bound endoglin counteracts these actions, thereby promoting angiogenesis. Cell-bound endoglin antagonizes the inhibitory signaling of TGF-β1 on human vascular endothelial cells, and normal levels of endoglin are required for formation of new blood vessels (139). Endoglin is overexpressed on highly proliferating endothelial cells in culture (138). In immunohistochemistry studies, CD105 is strongly expressed in blood vessels of tumor tissues in breast, colon and prostate cancer, brain and cervical cancer (140).

Microvessel density (MVD) is an immunohistochemistry based method, and determines neovascularization in tumor tissue with use of immunohistochemical markers for endothelial cells. MVD is assessed in the tumor areas with the greatest density of stained capillaries, and the number of vessels per high-power microscopic field is counted (141). Endoglin (CD105) positive immunohistochemistry has been used to assess intratumoral MVD, and several studies have shown that endoglin reflects neo-angiogenesis in tumor vasculature better than panendothelial markers, such as von Willebrand factor (vWF, also named factor VIII-related antigen), CD34 and CD31 (140). In ovarian cancer, endoglin

(CD105) was found to be an independent predictor of poor survival, and endoglin was a better predictor for MVD than CD31 (141).

Circulating endoglin (sEng, soluble endoglin) represents a soluble form of the cell-bound endoglin protein. sEng is an N-terminal cleavage product of full-length Eng but lacks the cytoplasmatic tail and transmembrane domain (142). Previous studies have shown that sEng correlates with metastasis and poor survival in colorectal, breast and lung cancer (143). Plasma levels of sEng correlate with metastasis in women with breast cancer, and the protein might be an angiogenetic marker for identifying breast cancer patients who are at high risk for developing metastasis (144). To the best of our knowledge, there is no published study exploring soluble endoglin in epithelial ovarian cancer.

5. AIMS OF THE THESIS

More knowledge regarding signaling pathways in the malignant transformation is important to improve both ovarian cancer diagnosis and treatment. The overall aim of the present thesis was to explore some molecular markers possibly involved in ovarian carcinogenesis, and to investigate whether BOTs and invasive carcinomas differentially express these biomarkers. The study also aimed at evaluating the current surgical treatment of BOTs.

Specifically, the aims were to:

1. Evaluate retrospectively surgical techniques (laparoscopy versus laparotomy) in women operated for BOTs, assessing preoperative, perioperative and postoperative findings. We also wanted to reevaluate histologically the BOT diagnoses, including the diagnoses of all benign or malignant ovarian tumors operated at Department of Obstetrics and Gynaecology, Ullevål University Hospital.

2. Analyze the expression of the AP-2γ transcription factor in BOTs, early-stage ovarian carcinoma and advanced-stage ovarian carcinoma, and to evaluate its prognostic role in advanced-stage tumors.

3. Study the expression of the nerve growth factor receptors TrkA and p75 in ovarian BOTs, early-stage ovarian carcinoma and advanced-stage ovarian carcinoma.

4. Assess a possible association between expression of the nerve growth factor receptors TrkA and p75 and mitogen-activated protein kinase (MAPK) in ovarian BOTs and FIGO stage I carcinomas.

5. Explore whether calprotectin, an inflammation marker, can be used as a plasma or effusion biomarker of ovarian cancer.

6. Investigate whether endoglin (CD 105), a protein highly expressed in tumor neoangiogenesis and elevated in the circulation in some cancer forms, can be used as a biomarker for ovarian cancer, either in plasma or in effusions.

6. MATERIALS AND METHODS

6.1 Patient selection

Biological material from 3 different biobanks (A-C) was used in Papers II-IV:

Biobank A is the GSI (Gynecological tumors- svulster in Norwegian- and Invasion potential) study biobank at Ullevål University Hospital, and was used in Papers III and IV. This biobank was established in 2003 by the Ullevål research group (Associate Professor Annetine Staff, Professor Mathias Onsrud, and MD Elin Ødegaard) and is an ongoing biobank collection, including surgical material from women operated for ovarian tumors (benign tumors, BOTs and invasive ovarian carcinomas) at the Department of Obstetrics and Gynaecology, Ullevål University Hospital. The collection of biological material and pre-, peri-and postoperative clinical information was collected for use in this thesis, from January 2003 until 2006. Serum and EDTA-plasma blood samples were obtained preoperatively from all the patients included in Papers IV and V. All tumors from women included in Papers IV and V from the GSI study were reevaluated histologically by Consultant Vibeke Engh, a senior pathologist at Ullevål University Hospital. Only the *blood samples*, not the tumor tissue, of the patients recruited from the GSI biobank were used for analyses in Papers IV and V.

Biobank B is an effusion and tumor biobank at the Department of Pathology, Norwegian Radium Hospital, Rikshospitalet Medical Center (in this thesis abbreviated to the RH biobank). The biological specimens were collected from 1991 to 2002, under the supervision of Associate Professor Ben Davidson.

Effusions

Ascites and pleural effusions from women with FIGO stage II-IV carcinomas were used in Paper II (168 women), and in Paper V (164 women). We did not have available effusions from women with benign ovarian tumors, as these rarely produce ascites or pleural effusions (except for Meig's tumors).

Tumor tissue

In Paper II, the tumor material consisted of sections from patients included in the RH biobank; 14 benign ovaries from patients operated for non-metastatic endometrial

carcinomas, 31 BOT, 22 primary stage I ovarian carcinomas, 42 primary advanced-stage (FIGO III-IV) carcinomas (40 serous, 1 undifferentiated and 1 mixed epithelial tumor) and 62 solid metastases. Tissue microarray (TMA) sections for c-kit immunostaining were from 35 patients with advanced-stage carcinomas (primary tumors, solid metastases and effusions). These 35 patients were part of the cohort studied for AP-2γ protein expression in Paper II. In Paper III, sections from 75 BOT, 57 FIGO stage I carcinomas and 56 advanced-stage carcinomas (24 primary and 32 metastatic lesions) from the RH biobank were used. All tumors from the RH biobank were histologically reevaluated by a senior pathologist (Ben Davidson). Borderline tumors were additionally evaluated by another senior pathologist (Vera Abeler).

Biobank C is a tumor biobank from the Department of Pathology at Sheba Medical Center in Israel. The tumor samples were from 44 paraffin blocks of borderline ovarian tumors and were used in Papers II and III. All tumor slides were diagnostically reevaluated by the same two senior pathologists as for Biobank B (Vera Abeler and Ben Davidson).

Surgical borderline ovarian tumor (BOT) patient population

In Paper I, we performed a retrospective comparison of short time outcome after surgery by laparoscopy or laparotomy in women with stage I ovarian borderline tumors during a five-year period (January 2000 to December 2004). In this period, 603 women with ovarian tumors were operated at the Department of Obstetrics and Gynaecology at Ullevål University Hospital, and of these, the 112 identified patients with BOT were included in the retrospective study. All tumors were reevaluated histologically by the same senior pathologist at Ullevål University Hospital as in Papers IV and V (Vibeke Engh). The medical records of all 112 patients with BOT stage I disease were studied retrospectively, supplemented by information from the patient's gynecologist or from the patients themselves if the last consultation was outside the hospital.

Ethical approval

The GSI biobank study (Papers IV and V) and the BOT operative study (Paper I) were both approved by the Regional Committee of Medical and Health Research Ethics in Eastern Norway and informed written consent was obtained from each patient. The effusion and tumor study (Papers II, III and V) from the RH biobank was approved by the Regional

Committee of Medical and Health Research Ethics in Southern Norway. Local ethical consent was obtained for the Israeli study in Israel. Norwegian regulatory rules and laws, including Data Inspectorate regulations and Biobank law, were followed strictly, including the handling of the Israeli tumor biobank tissue (evaluated and transported anonymously).

6.2 Laboratory methods

Preparation of effusions

Fresh (non-fixed) peritoneal and pleural effusion from ovarian cancer patients were obtained from Biobank B (RH biobank). All effusions were morphologically evaluated by an experienced cytopathologist (Associate Prof. Ben Davidson). All specimens containing degenerated cells were excluded from the study. Specimens that contained a large amount of viable cells (malignant-appearing epithelial cells, mesothelial cells, or both) were processed immediately after tapping. Part of the material was fixed in formalin and used for the preparation of a cell block (Paper II). The other part of the material was centrifuged at 2000 rpm for 10 minutes, and separated into cell pellets (used in Paper II) and supernatants (used in Paper V). Both were frozen at –70^{o}C.

Immunohistochemistry (IHC)

Four to six micron thick sections from ovarian tumors were formalin–fixed, paraffin–embedded and mounted on coated slides for IHC (Papers II and III). The slides were immunostained using the DAKO EnVision ^{TM} + peroxydase system (DakoCytomation, Glostrup, Denmark). Deparaffinized sections were microwaved in buffer, as described in Papers II and III, in order to unmask the epitopes, and then treated with hydrogen peroxide (H2O2) for 5 minutes for blocking endogenous peroxidase. Incubation was done with mono- or polyclonal rabbit antibodies (see Papers II and III). The sections were then incubated with the respective antibodies followed by incubation with the secondary antibody, the peroxidase-labeled polymer conjugated anti-rabbit or anti-mouse, for 30 minutes. Tissues were stained for 5 minutes in freshly prepared diaminobenzidine terahydrochloride (DAB), and then counterstained with haematoxylin, dehydrated and mounted on slides. All series included positive controls, consisting of sections from invasive ovarian carcinoma tissue that was shown to express the protein in a pilot study. The exception to this was the c-kit positive control, which was an appendix specimen. All series included negative controls, performed on sections from the same tumor tissue as the positive

controls; the negative controls underwent similar staining procedure, except for the primary antibody application. Most of the IHC in Papers II and III was performed by the PhD student Elin Ødegaard, the remaining slides were stained by two technicians, Inger Liv Nordli and May Nguyen.

Western blots

Cells from Biobank B (RH biobank) effusions and ovarian carcinomas cell lines (SKOV-3 and OVCAR-3) were studied for AP-2γ expression (see Paper II). This Western immunoblotting was performed by Martina Skrede. Totally, 61 (45 peritoneal, 16 pleural) malignant effusions from advanced-stage ovarian carcinoma patients and the ovarian carcinoma cell lines SKOV-3 and OVCAR-3 were studied for AP-2γ expression. Frozen samples were thawed, washed twice in cold PBS, and lysed in NP-40 ice-cold lysis buffer (1% NP-40, 10% glycerol, 20mM Tris-HCl [pH 7.5], 137mM NaCl, 100mM NaF, 1mM sodium vanadate, 1mM phenylmethyl sulphonyl fluoride, and 0.02mg/ml each of leupeptin, pepstatin, and aprotinin, and 10µl/ml phosphatase inhibitor cocktail I). All inhibitors were from Sigma-Aldrich, St. Louis, MO. Lysates were then sonicated and centrifuged. Protein was quantified by Bradford analysis and 25 micrograms of total cellular protein were loaded into each lane, separated by electrophoresis through SDS-12% polyacrylamide gels (SDS-PAGE), blotted onto immobilon-P-membranes (Millipore Corporation, Bedford, MA) and blocked in 5% non-fat dry milk in TBS-T. The filters were subsequently hybridized with the antibody against AP-2γ used for IHC, using a concentration of 1µg/ml. A mouse monoclonal antibody against α-tubulin (clone 57) (Oncogene, San Diego, CA) was used as loading control at a concentration of 1:5000. After washing in TBS-T, bound antibody was visualized using peroxidase-conjugated anti-mouse IgG and the ECL detection system (Amersham Pharmacia, Buckinghamshire, UK). Negative controls consisted of antibody in the absence of lysate.

ELISA

Enzyme-linked immunosorbent assay method was used for protein measurement of calprotectin and endoglin in plasma and ascites, and was mainly performed by technicians. ELISA for **calprotectin** was performed at the Department of Immunology and Transfusion Medicine, Ullevål University Hospital (Paper IV) on plasma samples from Biobank A and on effusion samples from Biobank B (Paper V). A plasma volume of 100 uL was diluted

1/50 in an assay buffer containing 10g/L bovine serum albumin, 50 mmol/L tris, 150 mmol/L sodium chloride, 0.5 mmol/L magnesium chloride, 2.5 mmol/L potassium chloride, 0.25 mmol/L thimerosal, and 0.05% Tween-20, pH 8.0. The standards and diluted samples were added to microtiter wells and shaken for 30 minutes at room temperature. After washing, alkaline phosphatase-conjugate was added, and the plate was shaken again. After a final wash, substrate was added and the optical density was read. Coefficients of variation were 5% within and 13% between assays.

Enzyme-linked immunosorbent assay for human **endoglin** (Paper V) was performed in duplicates on plasma samples from Biobank A and on effusion samples from Biobank B, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Routine blood analyses

High-sensitivity C-reactive protein (**CRP**) was measured in serum (Paper IV) with a Hitachi 917 instrument by particle-enhanced immunoturbidimetric assay (Tina-Quant CRP, Roche Diagnostics Corporation, Indianapolis, IN), measuring the range of 0.1-300 mg/L.
White cell count was assessed using a Sysmex SE 9500 (TOA Medical Electronics, Kobe, Japan).

Serum CA 125 concentration (Papers I and IV) was measured with an ARCHITECT system using a two-step immunoassay technology (Abbot Laboratories, Abbott city, IL).

6.3 Statistics

The results in Paper I to V were usually not normally distributed. Median and range or median and a 95% confidence interval of the median were reported. In Papers I, II, IV and V we used Mann-Whitney test when analyzing numerical or continuous variables. In Paper V we also performed the Kruskall-Wallis test (for > 2 categories, e.g. histological grade). In Papers I-V, categorical variables were analyzed by Chi-square test or Fisher's exact test (when the expected values were less than five in a 2x2 table). Comparative analysis of the AP-2γ protein expression (Paper II) in primary and metastatic advanced-stage carcinomas was performed using the Wilcoxon signed ranks test. Univariate survival analyses for patients with advanced-stage tumors were analyzed by the Kaplan-Meier method (Paper II and Paper V). In Paper IV, Receiver Operating Characteristic (ROC) curves were

constructed for calprotectin and CA 125 evaluations. In Papers IV and V, we also performed Spearman's correlation to calculate correlation coefficients. The statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS, version 13-14, Chicago, IL). A significance level of 5% was used.

7. SUMMARY OF RESULTS

Table 1 gives an overview of the results of this thesis regarding tissue-investigated, plasma- and effusion-investigated potential protein biomarkers for women with ovarian tumors (Papers II-V).

Paper I: Surgery of borderline tumors of the ovary: retrospective comparison of short-term outcome after laparoscopy or laparotomy

All ovarian tumors operated during a 5-year period at our Department of Obstetrics and Gynaecology (January 2000 through December 2004) were reevaluated histologically by one senior pathologist. Of the 603 tumors, only 3 (0.5%) were reclassified during the histological revision, with 1 benign cyst being upgraded to BOT, 1 BOT being downgraded to benign cyst, and 1 invasive carcinoma being downgraded to BOT. In the histological reevaluation of the 603 patients, 19% were classified as BOT (n=107), 33% as carcinomas and 48% as benign ovarian tumors.

In Paper I we also retrospectively evaluated the short-term outcome for the 107 women operated in this 5-year period for BOT, FIGO stage I, comparing two different surgical approaches (laparoscopy and laparotomy). Women who initially underwent laparoscopy, but during the operation had a conversion to laparotomy, were analyzed in the laparotomy group. Women operated by laparotomy in a first operation (n=69) were more often postmenopausal and had a higher CA 125 preoperatively than women operated by laparoscopy (n=38). The median tumor size was larger in the laparotomy group compared to the laparoscopy group (16.4 cm versus 8.6 cm, $p<0.001$). Tumor rupture during operation was more frequent in the laparoscopy group, 29% versus 16%, but this difference was not statistically significant ($p=0.2$). In a sub-analysis of tumors exceeding 10 cm in diameter, we found significantly more frequent rupture with laparoscopy than laparotomy ($p=0.014$). Postoperative complications were analyzed combining primary and a potential second operation in the laparotomy group (n=73), and were more frequent than in the laparoscopy group (18% versus 3%, $p=0.03$), wound hematoma representing the most frequent complication. Median hospital stay was also longer for patients operated by laparotomy (5 versus 1.7 days, $p=0.001$). The laparoscopic surgeries were however less extensive as compared to laparotomy surgeries, and did not include hysterectomy.

Paper II: The AP-2γ transcription factor is upregulated in advanced-stage ovarian carcinoma

AP-2γ transcription factor seems to be involved in the regulation of many factors that are altered in cancer, such as proliferation, cell cycle regulation, inhibition of apoptosis and invasion, but their exact role in tumor genesis is not established. In Paper II, sections from 14 normal ovaries, 75 BOT, 22 FIGO stage I ovarian carcinomas and 306 advanced-stage (FIGO II-IV) ovarian carcinomas (42 primary tumors, 62 metastases and 202 effusions) were analyzed for expression of the transcription factor AP-2γ by immunohistochemistry. Also, 63 effusions and 2 cell lines (SKOV-3 and OVCAR-3) were studied using immunoblotting. AP-2γ was detected predominantly in the nucleus of BOT (37%), FIGO stage I carcinomas (59%), and primary tumors from advanced-stage carcinomas (93%), and in cancer cells of effusions from women with advanced-stage carcinomas (79%). The surface epithelium of benign ovaries did not express the AP-2γ protein by immunohistochemistry. AP-2γ nuclear expression was upregulated in advanced-stage ovarian carcinomas ($p<0.001$) compared to stage I carcinomas and BOT. AP-2γ expression did not correlate with protein expression of the tyrosine kinase receptor c-kit, although these proteins have previously been shown to be co-expressed in melanoma. Analysis from corresponding primary tumors, metastasis and effusions from advanced-stage cases showed no difference in AP-2γ nuclear expression ($p>0.05$). AP-2γ expression in primary tumors of advanced-stage, solid metastases or effusions did not correlate with progression-free or overall survival time period for the included women.

Paper III: The activated nerve growth factor receptor p-TrkA is selectively expressed in advanced-stage ovarian carcinomas

We wanted to analyze the association between NGF receptor expression and the malignant potential in ovarian tumors, as NGF induces cell proliferation via activation of TrkA and MAPK in breast cancer cell lines. We compared the expression of the NGF receptors p-TrkA (a high-affinity receptor) and p75 (a low-affinity receptor) in borderline tumors and invasive ovarian carcinomas. Sections from 119 BOT, 57 FIGO stage I ovarian carcinomas, and 56 advanced-stage ovarian carcinomas were immunostained for p-TrkA, p75 and the activated forms of MAPK (p-ERK, p-P38 and p-JNK). We found an up-regulation of p-TrkA in advanced-stage carcinomas as compared to stage I carcinomas ($p<0.001$). There was no significant difference in p-TrkA protein expression between BOT and stage I

ovarian carcinomas. p75 results showed low membrane expression in all 3 groups, and no difference between the BOT, early- and advanced-stage ovarian carcinomas ($p>0.05$). MAPK nuclear protein expression was very high in both the BOT and stage I carcinoma group, but there were no statistical differences between these tumor groups with respect to p-ERK or p-P38 expression. P-JNK was more frequently expressed in stage I ovarian carcinoma compared with BOT ($p<0.001$). We found no association between NGF receptor expression and MAPK activation in BOT and stage I carcinomas.

Paper IV: Circulating calprotectin in ovarian carcinomas and borderline tumors of the ovary

We included 199 women from our biobank research study (GSI) at Ullevål University Hospital. Calprotectin, a marker of inflammation, may be involved in tumor development, but the exact biological role remains to be defined. Calprotectin was analyzed using ELISA in EDTA-plasma collected prior to surgery from women with ovarian carcinomas (n=89), borderline ovarian tumors (BOT, n=39) and benign ovarian tumors (n=71). Median plasma concentration of calprotectin was significantly elevated in women operated for invasive cancer (2512 µg/L) compared to borderline (985 µg/L) and benign tumor groups (951 µg/L) (both $p<0.001$). Median plasma calprotectin concentration did not differ between the BOT and benign control groups. Serum CA 125 was analyzed in the same study population. A positive correlation was found between CA 125 and calprotectin concentrations in ovarian carcinoma (Spearman's correlation: 0.5, $p<0.05$). In order to explore whether calprotectin could represent an improved marker as compared to CA 125 in identifying ovarian cancer, we used a receiver operating characteristic (ROC) curve, plotting the sensitivity versus 1-specificity for each possible cut-off. The ROC curve demonstrated a larger area under the curve for CA 125 (0.85) as compared to calprotectin (0.70), suggesting that plasma calprotectin is inferior to CA 125 when used as an ovarian cancer biomarker.

Paper V: Endoglin and calprotectin as potential biomarkers in ovarian carcinoma and borderline tumors of the ovary

In this study we used the same GSI biobank plasma material as in Paper IV, including women with benign ovarian tumors (n=71), BOT (n=39) and ovarian carcinomas (n=89). In addition, we included effusions from 164 women with advanced-stage ovarian carcinoma. Plasma samples and effusion supernatants were analyzed for endoglin by ELISA.

Calprotectin was analyzed in the effusion specimens by the same immunoassay as in Paper IV. Unexpectedly, a slightly higher median plasma concentration of endoglin was found in the BOT group as compared to both control and invasive cancer group (4.9 versus 4.5 and 4.3 ng/mL, p=0.02 and p=0.04). Median plasma endoglin concentration did not differ significantly between the control and invasive groups (4.5 vs 4.3 ng/mL, p=0.08). For the total group, there was no correlation between serum CA 125 and plasma endoglin concentrations. In effusions, calprotectin had a medium concentration of 2509 μg/L and median calprotectin concentration was significantly higher in effusion obtained prior to chemotherapy administration compared to post-chemotherapy specimens (p=0.007). Endoglin concentration in effusions supernatants had a medium value of 3.6 ng/mL, which was of corresponding magnitude to the median plasma concentration, although measured in different patient populations. Endoglin and calprotectin effusion concentrations did not correlate with overall or progression-free survival, or showed any association with histological grade, FIGO stage or extent of residual disease (all p>0.05).

Biomarker	**Laboratory method** (and biological material)	**BOT**	**Ovarian carcinoma** (FIGO I)	**Ovarian carcinoma** (FIGO III-IV)	**Effusions** (FIGO III-IV)	**Paper**
Ap-2γ (transcription factor) [b) c)]	**IHC** (paraffin sections) **Western** (malignant effusions)	↔	↔	↑	↑ frequent expression No correlation with survival	**II**
TrkA (nerve growth factor) [b) c)]	**IHC** (paraffin sections)	↔	↔	↑	↑	**III**
MAPKs (intracellular signaling pathways) [b) c)]	**IHC** (paraffin sections)	↑	↑			**III**
Calprotectin (inflammation marker) [a) b)]	**ELISA** (plasma) (effusions)	↔	↔	↑	No correlation with survival	**IV** **V**
Endoglin (angiogenic factor) [a) b)]	**ELISA** (plasma) (effusions)	(↑)	↔	↔	No correlation with survival	**IV** **V**

Table 1: Summary of results in Papers II-V

As compared to controls: ↔: no significant difference, ↑: higher in, ↓: lower in

[a)] GSI biobank (biobank A)

[b)] RH biobank (biobank B)

[c)] Israeli biobank (biobank C))

8. GENERAL DISCUSSION

8.1 Patient selection

Population used in Paper I

In Paper I we used a retrospective design to compare the two different surgical methods in women operated for stage I BOT, laparoscopy and laparotomy, in a five-year period from January 2000 to December 2004. The study design would be improved if we had performed a randomized prospective study, where neither the gynecological surgeons nor the patients would be able to influence the choice of operating method. An improved design would be a multicenter study, including a much larger patient cohort, giving us the possibility to match the patient operation groups for age, BMI, fertility wishes, and risk for malignancy index (RMI). Selecting patients for a prospective operative study for borderline ovarian tumors would however include the risk of including some patients with early ovarian invasive cancer, as well as women with benign ovarian tumors. The final histological diagnosis of BOT is available days or weeks after the operation, after the final formalin-fixed, paraffin-embedded sections have been analyzed by a pathologist, and intra-operative frozen section diagnosis is often difficult to rely on, especially for the mucinous histology.

Population used in Papers II-V

In the thesis, biological tissue from 3 different biobanks (A-C, see Chapter 6) was used. In Papers II and III, **Biobank C** includes Israeli biobank sections from Israeli women with BOTs. These specimens were obtained through collaboration between Ben Davidson and the Sheba Medical Centre, and limited clinical information was available regarding the patients. In the Jewish population, the Ashkenazi Jewish carry a BRCA mutation in 1 of 40 individuals, and up to 40% of ovarian cancer in this population is believed to be of hereditary nature (145). In the Norwegian population, BRCA1 mutations seem to be less prevalent, about 4% (146). In Norway, BRCA2 mutations have been demonstrated to have no significant association with inherited epithelial cancer (147).

Blood sample analyses from **Biobank A**, the GSI biobank study, were included in Papers IV and V. The plasma sampling started January 2003, including women with suspected ovarian tumors operated at the Department of Obstetrics and Gynaecology, Ullevål University Hospital in this prospective GSI biobank study. The patients signed informed consent, if

willing to be recruited. Only 2 of 365 patients have until January 2008 refused to participate in the study, when invited. The Biobank includes personal interview with each woman, thorough review of the patient's medical record, preoperative blood sampling, snap freezing of tumor tissue, preparation of paraffin blocks of formalin fixed primary tumor and metastatic tissue, and collection of ascites and pleura effusions, if present. We believe that the recruited patients are representative of all patients operated at the Department, as only random PhD/technician availability on regular working days is affecting the capacity to include all patients operated.

As for the recruited population representing the general population of the Oslo area, our study may tend to over-represent women with BOT and benign tumors and under-represent women with advanced ovarian cancer. In Norway, there is a principle of free patient choice of which hospital to be treated at. In emergency settings, and for most patients in practice, the local or regional hospital will be the one to serve the patient. Ullevål University Hospital is the largest hospital in Norway and the Department of Obstetrics and Gynaecology serves as a local hospital for Oslo patients as well as serving as a regional gynecological oncology unit for the Regional Health Authority of Eastern Norway (until June 2007). The Norwegian Radium Hospital (now part of the Rikshospitalet Medical Center) served as the regional gynecological oncology unit for the Regional Health Authority of Southern Norway (until June 2007). Women with obvious ovarian cancer, when examined by gynecology specialists outside hospital, tend to get referred to the Norwegian Radium Hospital. This center does not serve as a local hospital and does not treat women with benign ovarian pathology.

Table 2 shows the number of patients included in the GSI biobank at Ullevål University Hospital each year (and the percentage of women included in the GSI biobank of the total amount of women operated at Ullevål University Hospital for the same diagnoses). Table 2 also shows the number of patients operated at the Norwegian Radium Hospital in the same period.

As seen from Table 2, 87% of all women operated for BOT were included in the GSI biobank study at UUS in 2004, 67% were included in 2003, whereas only 14% were recruited in 2005 (when the PhD student EØ was primarily performing IHC and not recruiting study patients).

N= patients operated	**2003**	**2004**	**2005**	**2006**
Ullevål University Hospital (GSI, % included of all operated)				
BOT	12/18 (67%)	20/ 23 (87%)	3/22 (14%)	6/19 (32%)
Invasive epithelial ovarian carcinomas	24/37 (65%)	39/55 (71%)	15/42 (36%)	22/64 (34%)
Norwegian Radium Hospital				
BOT	28	29	22	24
Invasive epithelial ovarian carcinomas	176	199	162	156
Norway				
BOT	176	176	168	155
Invasive epithelial ovarian carcinomas	381	416	385	400

Table 2: The numbers of women operated for BOTs and invasive carcinomas at Ullevål University Hospital, at the Norwegian Radium Hospital and in Norway between 2003-2006 (Norwegian Cancer Registry).

Histological reevaluation of the diagnoses

All tumors used in this thesis were histologically reevaluated by one or two senior pathologists. In Papers I, IV and V, a senior pathologist at Ullevål University Hospital, Vibeke Engh, reevaluated all included malignant and borderline tumors. In Paper I, all benign cysts and tumors operated in this period (January 2000 to December 2004) were also reevaluated by Engh. In Paper I, only 3 of 603 histological diagnoses were reclassified following reevaluation. In a large population-based study from another health region in Norway during a 10-year period, the accuracy of the ovarian cancer diagnosis (without BOT included) was estimated as 92%, when reevaluated by a senior pathologist (in 591 patients primarily diagnosed histologically with ovarian cancer) (148). Stalsberg et al. published a study in 1988, reviewing 869 tumors primarily diagnosed as malignant or BOT, as reported to the Cancer Registry of Norway from different hospitals in Norway. The histological slides were randomly distributed to six pathologists and classified according to the WHO classification of ovarian tumors, demonstrating a mean intra-observer reproducibility of 62%. The most common disagreement was in identifying the specific type of carcinoma, differentiating between undifferentiated and differentiated carcinomas as well as between borderline and malignant tumors. Very low reproducibility was obtained for diagnosing mixed and unclassified carcinomas (149).

In the present thesis, all specimens used were reevaluated by senior pathologists who are specialists in gynecopathology, including Vera Abeler and Ben Davidson for Biobank B and C (Papers II, III, IV and V), whereas Vibeke Engh reevaluated specimens from Biobank A (Papers IV and V) and tumors from women included in Paper I.

Long-term follow-up

In the retrospective borderline study (Paper I), we were not able to do any long-term follow-up because the women were recently operated (January 2000 to December 2004). If we had included women operated twenty years ago, long-term follow-up would have been possible, but laparoscopic advanced surgery was not an option at that time. Patients with BOTs have an excellent prognosis (150), and therefore the observation time in this study is too short to conclude regarding long-term aspects. Long-term follow-up was also not possible for women included in Papers IV and V, as the prospective GSI study has a short observation time, including women in the study from January 2003 to December 2006.

Controls

In Papers IV and V, the selection of control patients could be questioned, comprising women being operated for benign ovarian masses. We believe that this is a relevant clinical group to include as controls in our study, rather than using a group of healthy women without ovarian tumor. Preoperatively, for a woman presenting with an adnexal tumor, a blood-based biomarker would be helpful in discriminating between benign ovarian tumors, BOT and invasive ovarian cancer, in addition to the clinical and ultrasound findings. In the evaluation of endoglin and calprotectin as potential biomarkers in effusions (ascites and pleural effusions), we included no benign controls. Benign ovarian tumors very seldom produce effusions, compared to advanced-stage ovarian cancer, where the metastatic spread often is by excessive effusion production by cancer cells.

8.2 Methodological considerations

Specific antibodies are indispensable tools when investigating protein expression in tissue and effusions. Immunohistochemistry (IHC) is simple and is part of the armament of clinical pathology laboratories. Our aim was to investigate potential early markers of ovarian cancer, as well as potential prognostic markers in advanced-stage disease (Papers II and III). IHC may differ between laboratories as function of different procedures, different

antibodies, different staining protocols and different scoring systems. In Paper II we therefore additionally performed immunoblotting as a validation method. In Paper III only immunohistochemistry was performed for protein assessment, as immunoblotting had been done on a part of this material in another study and had already shown strong p-TrkA expression.

In Papers IV and V we used ELISA to analyze the calprotectin and endoglin proteins both in the circulation (plasma) and in effusions (ascites/pleural effusions). We could have used other methods such as immunoblotting to confirm the results, giving additional information on protein size detected, but Western blot is more time consuming and less reproducible quantitatively than commercially available ELISA. Other investigators (151) and our group at Ullevål University Hospital (152) have previously used the same soluble endoglin kit used in Paper V, detecting high concentrations of sEng. The cell-bound endoglin (CD 105) protein has also been detected by immunohistochemistry in ovarian cancer in a study by Taskiran (141). Calprotectin has been found elevated in plasma from patients with several cancer forms, including colorectal cancer (134), using the same ELISA method as in this thesis (Papers IV and V).

8.3 Sample size calculations

We did not perform detailed sample size analyzes prior to the start of the different studies, as relevant data on the investigated proteins in the tumor tissue (Papers II and III) or in the circulation (Papers IV and V) were lacking, and therefore no standard deviations were available. The studies in this thesis could therefore be considered as pilot studies. In future studies, our results could be used for calculations of sample size.

In our studies, a too small sample size is especially relevant for the very small FIGO stage I group (n=10) included in Papers IV and V. Both type I (false positive result) and type II error (false negative result) may occur when including small populations. Also, we performed multiple statistical tests in all papers (Papers I-V). We could have performed a statistical correction, such as Bonferroni's correction, to reduce the risk of type I error. This was done in Paper V (see comments section), and almost all differences between groups remained statistically significant.

8.4 Interpretation of the results

Paper I: Laparoscopy versus laparotomy surgery in borderline tumors of the ovary
There is agreement on surgical removal of BOT as the cornerstone in the management of this tumor. Over the last decades, the management of BOT has changed from radical surgery to more conservative therapy, with more focus on the excellent prognosis for most BOT patients, and focus on preservation of fertility for younger women with BOT. There have been improvements and increasing use of laparoscopy in surgery in general, as well as in oncological surgery.

In our study (Paper I), intraoperative tumor rupture was significantly higher when the tumor diameter exceeded 10 cm in diameter. Neither this study (Paper I) nor others previously reported (154;155) can conclude at which precise tumor size cut-off a laparotomy approach is recommended prior to laparoscopy for a malignant suspected tumor. Conservative surgery for stage I BOT with complete staging, including preservation of the uterus and at least one or a part of the ovary to preserve fertility, is reviewed in an article by Cadron et al. (15). Conservative surgery for stage I BOT is regarded safe, providing long-time follow-up with clinical examination and ultrasound. The use of cystectomy, instead of unilateral salphingo-oophorectomy, has a higher risk of relapse (155;156). In Paper I, the follow-up time is too short to conclude whether the choice of operating method has any influence on the prognosis. Although laparoscopy is gaining popularity in treatment of BOT, some gynecological oncology groups advocate that primary laparotomy is the preferred technique (15).

All published studies regarding laparoscopy versus laparotomy in BOT surgical treatment are retrospective and not randomized. Prospective randomized studies are needed to conclude on effects of surgery type on long-term survival and relapse rates.

Paper II: The AP-2γ transcription factor
AP-2γ transcription factor is involved in the tumorigenesis of ovarian carcinomas, although the exact role is not entirely elucidated. AP-2γ is not expressed in normal ovarian epithelium. The expression of nuclear AP-2γ in BOT and stage I carcinomas on tumor level is less frequent than in advanced-stage carcinomas. Although we have not explored the concentration of this protein in the patients' circulation in our study (for example using the

GSI study samples), we do not believe AP-2γ will have a place as a potential circulating marker for early-stage ovarian cancer.

We found that AP-2γ is expressed in nearly all advanced-stage primary tumors, as well as metastatic effusions. Because of its frequent expression we cannot use AP-2γ as a prognostic factor in the advanced group. AP-2γ transcription factor is frequently expressed both in the primary advanced-stage carcinomas and in the effusions, when the tumor has become metastatic. We do not know whether AP-2γ overexpression represents the crucial step of tumor metastasis. AP-2γ seems to have a dual role in cell regulation, and AP-2γ may contribute to tumor development once compensatory mechanisms are overcome (82;157). It is essential to evaluate AP-2 activity and the interacting cofactor network in order to highlight the biological role of AP-2 transcription factors in ovarian cancer. Expanded knowledge of different AP-2 proteins may offer new strategies for cancer diagnosis and management in the future (83).

Paper III: Nerve growth factor receptors could be a therapeutic target in ovarian cancer treatment

The activated nerve growth factor receptor p-TrkA was selectively expressed in advanced-stage ovarian carcinomas. We cannot use TrkA or p75 to segregate BOTs from FIGO stage I carcinomas by immunohistochemistry, since both tumor types had low expression of these receptors. The upregulation of p-TrkA in ovarian carcinomas is specific for the advanced-stage of disease, when the ovarian carcinoma has become metastatic. This suggests that upregulation of p-TrkA is one of the defining steps in tumor progression in ovarian carcinomas.

The generally high MAPK activation in stage I carcinomas and BOTs that we report in Paper III is in agreement with other studies in ovarian carcinoma where high MAPK activity has been associated with improved prognosis and less aggressive clinical behavior (106;108). Downstream targets of TrkA receptor activation may be along the PI3K/AKT pathway, which we will evaluate in further studies.

Since the TrkA receptor is overexpressed in a variety of human tumors and represent a marker of poor prognosis, drugs that target this receptor are being developed (158). These drugs inhibit autophosphorylation and signaling via Trk family members. CEP-751 may be

a useful therapeutic agent by inhibiting TrkA and inducing tumor apoptosis (158).

Papers IV and V: Calprotectin in preperative plasma in ovarian neoplasms and in effusions from advanced-stage carcinomas

In Paper IV, the patients were included from 2003 and the short observation time with little relapse and survival data makes it too early to evaluate the prognostic impact of plasma calprotectin concentrations. Plasma calprotectin was elevated in patients with invasive ovarian epithelial cancer, but used as a tumor marker it is inferior to CA 125. In our small group of patients with FIGO stage I ovarian cancer (n=15), 60% had elevated CA 125 (greater than 35 U/mL), as compared to 67% of the patients presenting preoperatively with elevated calprotectin plasma concentration (greater than 900 μg/L). Further studies are warranted, in stage I ovarian cancer disease, to assess whether calprotectin may serve as one of several biomarkers in a panel to improve preoperative diagnostics.

In Paper V, calprotectin was explored in 164 effusions from women with advanced-stage ovarian carcinomas. The concentration did not correlate with histology grade, FIGO stage or survival in advanced-stage ovarian cancer in effusions. Therefore, the clinical value of calprotectin effusion concentrations is at present unknown.

Paper V: Soluble endoglin

The exact mechanism for sEng formation is unknown. Although sEng correlates with metastatic disease in women with breast cancer (144), Paper V did not demonstrate a role for plasma endoglin in preoperative discrimination between benign and malignant ovarian disease in women presenting with adnexal tumor. Endoglin in effusions from women with advanced-stages of epithelial ovarian cancer did not correlate with survival or relapses in the patient cohorts.

Although we could not demonstrate a diagnostic preoperative role of plasma sEng in women with ovarian tumors, endoglin could bee explored as a biomarker for assessing effects of future antioangiogenic therapy in ovarian cancer (140;159).

9. CONCLUSIONS

1. Histological misclassification was very rare in the group of women operated for ovarian cysts and tumors at our Department of Obstetrics and Gynaecology (3 of 608 women being reclassified, Paper I). Our retrospective study showed that laparoscopic treatment of stage I BOTs compared to laparotomy is associated with fewer complications and shorter hospital stay. We also found that a BOT size exceeding 10 cm in diameter more often resulted in tumor rupture when using laparoscopic versus laparotomy approach (Paper I). Our retrospective study cannot conclude at which precise cut-off in tumor size laparoscopic versus laparotomy approach should be recommended in early-stage malignant suspect ovarian tumors. Peroperative tumor spilling can be reduced with the use of an endobag, and the bag size as compared to tumor size is one of the limiting factors to the laparoscopic approach, in addition to surgical expertise. Long-term follow-up of larger patient cohorts is necessary to determine the ultimate clinical outcome, especially after peroperative tumor rupture and spilling.

2. The transcription factor AP-2γ is immunohistochemically localized to the nucleus in ovarian BOTs and ovarian carcinomas, but not in the ovarian surface epithelium in normal ovaries (Paper II). AP-2γ expression is independent of the presence of the tyrosine kinase receptor c-kit in advanced-stage carcinoma. AP-2γ protein expression does not differ between BOT and FIGO stage I carcinomas. As protein expression of AP-2γ was significantly upregulated in advanced-stage tumors and effusions from stage III-IV carcinomas compared to stage I carcinomas, we suggest that this transcription factor is involved in tumor progression in ovarian cancer. AP-2γ protein expression did not predict survival in advanced-stage disease, and this may be related to its frequent expression (83%) in advanced-stage carcinomas (primary tumors, solid metastases and effusions).

3. The activated nerve growth factor receptor p-TrkA was selectively expressed in advanced-stage ovarian epithelial cancer (Paper III). p75 does not seem to be involved in tumor progression of ovarian carcinomas. The up-regulation of p-TrkA in advanced-stage disease of ovarian cancer could suggest that targeting this receptor may have therapeutic value for patients with advanced-stage ovarian disease.

4. MAPK activation is independent of p-TrkA and p75 expression in both BOTs and invasive carcinomas of the ovary, and we suggest that NGF receptors may signal via MAPK-independent pathways in ovarian carcinomas (Paper III). The downstream targets of p-TrkA and p75 receptors therefore remain to be identified, but we are currently evaluating the role of p-AKT in this setting. p75 appear to have no significant role in ovarian cancer biology or disease progression.

5. Plasma calprotectin concentration is elevated in women with invasive ovarian cancer compared to women with benign ovarian tumors and BOT, but the two latter groups do not differ in median plasma calprotectin concentrations (Paper IV). This study was too small to conclude whether calprotectin could represent an improved plasma-based biomarker for early-stage invasive carcinoma. In our patient population, calprotectin was slightly inferior to CA 125 in preoperatively differentiating between benign and malignant ovarian disease (Paper IV). Calprotectin concentration in effusions does not seem to have a predicting role for survival in advanced-stage ovarian carcinoma (Paper V).

6. Circulating endoglin is not elevated in advanced-stage ovarian carcinoma, in contrast to the situation in breast and gastric cancer (Paper V). Plasma endoglin concentration did not preoperatively discriminate between benign and malignant ovarian tumors in our study population. Also, effusion endoglin concentration does not seem to be related to survival in advanced-stage ovarian carcinoma.

More knowledge about signaling pathways in the malignant transformation in ovarian tumors is important in an era of targeted therapies. New biomarkers could be used for early detection of epithelial ovarian cancer, which is urgently needed to detect ovarian cancer at an earlier stage of the disease. Markers to validate treatment effects and to aid individualizing cancer treatment are warranted. Epithelial ovarian neoplasia comprises a heterogeneous group of tumors. There is no identified precursor lesion for ovarian cancer, and there is a lack of comprehensive tumor progression model. Our studies may have contributed to identify some small pieces of the enormous puzzle and enigma of ovarian carcinoma, a disease that deprives too many women of too many years of life.

10. FURTHER STUDIES

1. More biomarker studies on women with ovarian carcinoma, coupled to clinical data and survival

Our GSI biobank included by January 2008 biological samples and clinical information from more than 365 women. In addition to comprehensive clinical information, we have sampled preoperative blood samples, snap frozen (in liquid nitrogen) biopsies during surgery from the tumor and/or metastases as well as provided corresponding paraffin block from the same tumor. All biological samples have been carefully sampled and stored in a standardized way. The women included in the study have also given our research group at UUS the permission to follow them through hospital and death registries, and, if alive, to contact them again asking for participation in further research projects. This biobank enables several future projects, including studies on protein, mRNA as well as DNA levels. When looking at gene and protein expression from the same patients in different compartments (tumor, metastases, effusions, blood cells, serum/protein), we hope to identify new biomarkers, or combinations of such. Such new biomarkers may prove useful in identifying women with ovarian cancer as well as clinically segregate women with ovarian cancer into different prognostic and treatment modality groups.

2. Long-term survival of BOT patients according to surgical approach

Our Department of Obstetrics and Gynaecology treat surgically many women with BOT by laparoscopy. Women with BOT have an overall good prognosis. Longer observation time and systematic collecting of clinical findings would allow us in the future to evaluate the long-term effect of tumor spilling in the laparoscopic versus laparotomy approach on recurrence of disease. All BOT patients included in Paper I have signed informed consent to be followed clinically, also through other hospital and the Death and Cancer registries in Norway.

11. ERRATA

Paper I:

-Page 622: "All patients signed informed consents: Should read: "All BOT patients signed an informed consent…"

- Page 621: RMI has a reference number 7. The correct reference is number 8 (Tingulstad et al.)

Paper II:

- Page 463: Anttila et al. is correct, not Antilla et al.
- Page 464: Figure 1 text: Figure F, not E has a normal fallopian tube in the lower right corner
- Page 465: Table 2 heading: the study material consists of 403 specimens, not cases.

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